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Patterns of Variations in *Escherichia coli* Strains That Produce Cytolethal Distending Toxin

Carol L. Pickett,[1*] Robert B. Lee,[1] Aysegul Eyigor,[1] Ben Elitzur,[1] Emily M. Fox,[1] and Nancy A. Strockbine[2]

Department of Microbiology and Immunology, Chandler Medical Center, University of Kentucky, Lexington, Kentucky 40536-0298,[1] and Foodborne and Diarrheal Diseases Branch, Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, 30333[2]

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A collection of 20 *Escherichia coli* strains that produce cytolethal distending toxin (CDT) were analyzed for their virulence-associated genes. All of these strains were serotyped, and multiplex PCR analysis was used to ascertain the presence of genes encoding other virulence factors, including Shiga toxin, intimin, enterohemolysin, cytotoxic necrotizing factor type 1 (CNF1) and CNF2, heat-stable toxin, and heat-labile toxin. These CDT-producing strains possessed various combinations of known virulence genes, some of which have not been noted before. Partial *cdtB* sequences were obtained from 10 of these strains, and their predicted CdtB sequences were compared to known *E. coli* CdtB sequences; some of the sequences were identical to known CdtB sequences, but two were not. PCR primers based on sequence differences between the known *cdt* sequences were tested for their ability to detect CDT producers and to determine CDT type. Correlations between the type of CDT produced, the presence of other virulence properties, and overall strain relatedness revealed that the CDT producers studied here can be divided into three general groups, with distinct differences in CDT type and in their complement of virulence-associated genes.

The bacterial toxin cytolethal distending toxin (CDT) was initially described as having the ability to cause certain cultured cell lines to slowly distend and then die (18). More recently, several groups have shown that CDT causes sensitive cells to become apparently irreversibly blocked in either the G_1 (6, 14) or G_2 phase of the cell cycle (4, 32, 36). CDT apparently invokes this block by causing direct DNA damage through the action of the CdtB protein, which is a member of a class of phosphodiesterases that includes nucleases (8, 13, 19). The CdtA and CdtC proteins appear to be required as accessory proteins, mediating binding to the CDT receptor on sensitive cells (20, 21). CdtA and CdtC may also possess additional properties important for intoxication, but such roles have not yet been defined (12).

CDT is produced by several different bacterial species, including *Escherichia coli*, *Shigella* spp., *Campylobacter jejuni* and related thermophilic campylobacters, *Haemophilus ducreyi*, *Actinobacillus actinomycetemcomitans*, and enterohepatic *Helicobacter* spp. (5, 18, 31, 35, 37). In all of these species, CDT activity is encoded by three adjacent genes, *cdtA, cdtB*, and *cdtC*, and the expression of all three genes is required for the production of active CDT (31, 34). CDT production by *E. coli* strains was first documented by Johnson and Lior (18), and the *cdt* genes from three different *E. coli* strains were subsequently cloned and sequenced (29, 30, 34). These sequence data indicated that there is heterogeneity present in the *E. coli cdt* genes. For example, the predicted amino acid sequences of CdtA, -B, and -C from *E. coli* strains 9142-88 and 6468/62 have 48, 61, and 42% identical and conserved amino acids, respectively (30, 34). The third strain sequenced, S5, is most closely related to strain 9142-88, with CdtA, CdtB, and CdtC proteins that have 93, 94, and 94% identical and conserved amino acids, respectively (29). These CDTs have been called type I (strain 6468/62), type II (9142-88), and type III (strain S5), based on the order of publication of their gene sequences (29).

In this report, we have examined *cdt* sequences from a variety of *E. coli* strains in order to determine if additional sequence variants exist and to look for the possible association of specific types of *cdt* sequence variants with different *E. coli* virulence markers. Furthermore, we have tested the ability of PCR methods to detect and distinguish between the CDT types produced by these *E. coli* strains. Overall, our results show that these CDT-producing strains can be divided into three groups based upon their complement of virulence-associated genes and their type of CDT. In addition, we show the existence of two new *E. coli* CDT sequences that have not been previously reported.

MATERIALS AND METHODS

Strains, medium, and growth. Twenty *E. coli* strains that carry *cdt* genes as determined by PCR analysis (31) were used in this study. Nineteen of the strains were isolated from humans; one, strain F5953-98, was isolated from a calf. All of these strains but one were isolated from diverse geographical locations in the United States and Canada over the last several years and include several different serotypes (Table 1). Strain 3260-97 was originally isolated in Bangladesh. All of the strains, with the exception of H1, H19, and 2P6, were sent to the Centers for Disease Control and Prevention for serological or epidemiological analysis. Strains H1, H19, and 2P6, blood culture isolates from adult humans with urosepsis, were kindly provided by James R. Johnson and have been described by Johnson and Stell (17). *E. coli* strain S5 was used as a control in some experiments. It was kindly provided by Eric Oswald.

E. coli cultures were grown in L medium at 37°C at 250 rpm for 18 h. Strains were tested for CDT production according to the methods of Pickett et al. (30,

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Kentucky, Chandler Medical Center, 800 Rose St., Lexington, KY 40536-0298. Phone: (859) 323-5313. Fax: (859) 257-8994. E-mail: cpicket@uky.edu.

TABLE 1. *E. coli* strains used in this study and their relevant characteristics

Strain	O[a]	H[b]	CDT[c]	*stx1*[d]	*stx2*[d]	*eae*[e]	*Ehly*[f]	*cnf1*[g]	*cnf2*[h]
9142-88	128	NM	II	−	−	+	−	−	−
3102-97	R	NM	II	−	−	+	−	−	−
3260-97	R	NM	II	−	−	+	−	−	−
3032-96	119	NM	III	+	−	−	−	−	+
F6450-99	119	4	III	+	−	−	−	−	+
3139-97	88	25	III	+	+	−	+	−	−
3180-98	U	45	I	−	−	−	−	−	−
3015-99	2	NM	I	−	−	−	−	−	−
3061-99	18	1	I	−	−	−	−	−	−
H1	2	1	I	−	−	−	−	−	−
H19	2	NM	I	−	−	−	−	+	−
2P6	2	7	I	−	−	−	−	−	−
3008-99	2	NM	I	−	−	−	−	−	−
3142-98	63	4	I	−	−	−	−	−	−
3148-97	113	21	II/III	−	+	−	+	−	−
3084-98	158	NM	II[i]	−	−	+	−	−	−
F5953-98	126	8	III[j]	+	−	−	−	−	+
3014-99	88	7	II/III	−	−	−	−	−	−
F5971-99	U	25	II/III	+	+	−	−	−	−
F6448-99	73	18	II/III	−	+	−	−	−	−

[a] O antigen serogroup of the indicated *E. coli* strain. R, rough O antigen; U, O serogroup could not be determined with available reference sera.
[b] H antigen. NM, nonmotile.
[c] CDT type. II/III, strains could not be reliably designated as II or III since their *cdtB* genes were not sequenced.
[d] Shiga toxin gene, based on primers described by Olsvik et al. (26).
[e] Intimin gene; for *eae* and *eae* subtype determination, see references 1, 10, 22, and 33. Strains 3102-97 and 3084-98 were determined to be *eae*γ, but no *eae* subtype assignment could be made for 9142-88 or 3260-97.
[f] Enterohemolysin; primers were described in Fratamico et al. (11).
[g] Cytotoxic necrotizing factor type I gene, as reported in Johnson and Stell (17).
[h] Cytotoxic necrotizing factor type 2 gene; primers were described in Pass et al. (28).
[i] 3084-98 was designated type II on the basis of type-specific PCR only (see text and references 3 and 25).
[j] F5953-98 was designated type III solely on the basis of type-specific PCR (3).

31). All 20 strains produced CDT titers between 30 and 200; any differences did not correlate with CDT type, nor were they statistically significant.

PCR. An initial multiplex PCR analysis to determine the presence of diarrheagenic *E. coli* virulence genes was carried out as described previously (38). This analysis detects the presence of the *estI*, *eltI*, stx_1, stx_2, *eae*, *cnf1*, *Ehly*, *ipaH*, *uidA*, and *bfpA* genes and the EAF plasmid. Additional PCR tests were done to analyze for the presence of *cnf2*, *estII*, and F17 fimbriae (2, 27, 28). *eae* subtypes were determined by PCR analysis (1, 10, 22, 33).

PCR detection of *cdt* genes began with the use of the degenerative primers VAT2 and WMI1, according to the method of Pickett et al. (31). These primers are based on two highly conserved regions in *cdtB* and will amplify, in *E. coli* strain 9142-88, a 510-nucleotide *cdtB* fragment encoding 170 amino acids (30, 31).

Additional PCR analysis of the *cdt* genes included use of the reactions described by Okuda et al. (25) for detection of sequences related to the type I CDT sequence found in *E. coli* strain 6468/62 (34) and the type II CDT sequence from strain 9142-88 (30), as well as the PCR described by Clark et al. (3) for the detection of type III CDT sequences, based on that found in *E. coli* strain S5 (29).

Our type I- and type II/III-specific PCR analysis of the *cdtB* gene described in this work used four sequence-specific primers. Two of these primers were based on the type I *cdtB* sequence, and two were based on the type II *cdtB* sequence (30, 34). The type I primers were type I-F, 5′-GCTTCTGCAACGTT(G,T)ACTGAG-3′, and type I-R, 5′-AATCGCAAGATTTACTCTGTTAGCG-3′, and the type II primers were type II-F, 5′-TCAACGGCTGTA(A,G)ATACAGG-3′, and type II-R, 5′-CAGAGCAAGATTGACTCTTCCACCAAG-3′. These forward and reverse primers are based on amino acids 44 to 50 and 86 to 94 (Fig. 1), respectively. The forward primers were used in combination with the reverse primer WMI1; the reverse primers were used in combination with the forward primer VAT2. PCRs and cycle parameters were as described by Eyigor et al. (9), except that the annealing temperature was 47°C. All PCR primers were synthesized by Integrated DNA Technologies (Coralville, Iowa).

Randomly amplified polymorphic DNA (RAPD) PCR was performed using the Ready-to-Go RAPD analysis kit according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, N.J.). All six primers from this kit as well as primers 1247 and 1281 used for analysis of *E. coli* strains by Madico et al. (23) were tested for use in these studies. Primers 1, 2, and 3 from the Ready-to-Go kit proved to give the best range and distribution of product sizes.

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3032-96   VATWNLQGASATTESKWNINVRQLISGENAVDILAVQEAGSPPSTAVD    48
3260-97   ------------------------V-----------------------    48
3102-97   -----------------------------------I------------    48
9142-88   ------------------------------------------------    48
3015-99   --------SN-P--N---TH----VT-SG-----M-----AV-AS-TL    48

3032-96   TGRVIPSPGIPVRELIWNLSTNSRPQQVYIYFSAVDAFGGRVNLALVS    96
3260-97   -----------------------K------------------------    96
3102-97   -------Q---A------------------------------------    96
9142-88   --TL--------------------------------------------    96
3015-99   -E-EFST----MN-Y---TG------ELF----R----AN-----I--    96

3032-96   NRQADEVFVLRPVRQGGRPLLGIRIGNDAFFTAHAIATRNNDAPALVE   144
3260-97   ----------S--------------------------A------E---   144
3102-97   --R-----------------------------------------E---   144
9142-88   --R-------S--------------------------M----------   144
3015-99   --R----I--P-PTVVS--II-------V--ST--L-N-GV-SG-I-N   144

3032-96   EVYSFFRDSRDPVHQAINWMI                              165
3260-97   ---------------------                              165
3102-97   ---------------------                              165
9142-88   ---N-----------------                              165
3015-99   S-FE--NRQT--IR--A----                              165
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FIG. 1. Alignment of partial CdtB sequences. A comparison of the sequences of a portion of the predicted sequence of CdtB from five *E. coli* isolates is shown. The names of the strains are shown at the left. Strain 3032-96 makes a type III CDT, strains 3260-97, 3102-97, and 9142-88 make a type II CDT, and strain 3015-99 makes a type I CDT. The amino acid sequences are numbered continuously at the right. The initial valine shown is residue number 25 of the CdtB sequence of *E. coli* strain 9142-88 (30).

Cloning and sequencing. Total DNA was isolated from *E. coli* strains using a QIAamp tissue kit (Qiagen Inc., Valencia, Calif.), after which the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, Calif.) was used to amplify and clone a portion of the selected strain's *cdtB* gene. The degenerative sequences, VAT2 and WMI1, described above were used as primers in the amplification. At least two clones originating from each strain were sequenced (SeqWright, Houston, Tex.).

Nucleotide sequence accession numbers. The nucleotide sequences of the partial *cdtB* genes have been assigned GenBank accession numbers AY423896, AY423897, AY426340, and AY426341.

RESULTS

Analysis of *cdtB* sequences revealed invariant and variant CDT types. Multiplex PCR analysis for virulence factors commonly associated with *E. coli* disease indicated that *E. coli* strains identified as carrying the *cdt* genes apparently often carry additional virulence markers associated with either diarrheagenic or uropathogenic *E. coli* (Table 1). Based on these data, a portion of the sequence of the *cdtB* gene from 10 representative strains was amplified, cloned, and sequenced for comparison to already-known *E. coli cdtB* sequences. Translations of the partial sequences of five strains' *cdtB* genes are presented in Fig. 1. Strain 3032-96, whose complete amino acid sequence for the cloned region is shown, has a sequence that is identical to that of the type III CDT-producing strain, S5 (29). Two other strains, F6450-99 and 3139-97, also had this same sequence (data not shown). Another two strains, 3260-97 and 3102-88, had sequences similar to the S5 sequence and to the type II CDT-producing strain, 9142-88, (30), but several amino acid substitutions were present in the sequences in each of these strains (Fig. 1). The figure compares these substitutions to the sequences for both S5 and 9142-88; overall, the four sequences vary at 12 different residues within the 165-amino-acid *cdtB* fragment shown. Finally, strain 3015-99 represented five strains with identity to the type I strain, 6468/62, whose *cdt* gene sequence was reported by Scott and Kaper (34). One of these five strains, 3180-98, had a single amino acid substitution, Gly for Arg, at position 89. Overall, our results indicated the presence of three types of sequences that could be grouped into two broad divisions. First, there were those sequences that were identical or nearly identical to the type I sequence reported by Scott and Kaper (34). Secondly, there was a group of strains with partial CdtB sequences identical to that found in the type III CDT strain S5 (29). And lastly, there were two strains with partial CdtB sequences that were closely similar to that of both the type II strain 9142-88 (30) and the type III strain S5 (29) but which had at least five substitutions within the 165-amino-acid region of CdtB when compared to the prototype type II and III strains (Fig. 1). When referring to the latter two groups together, we have called them the typeII/III group. Individually, type II strains include the prototype type II strain 9142-88 as well as the two strains 3260-97 and 3102-97 that were similar but not identical to both 9142-88 and S5. Type III strains are those strains whose sequences were identical to that of S5.

Type-specific PCR for classification of *cdt* genes. Examination of the different partial *cdtB* sequences suggested that it would be possible to make PCR primers that were specific for either type I or for type II and III (II/III) *cdtB* sequences. We used four specific primers in combination with VAT2 or WMI1 to test whether we could use these primers in a screening PCR to determine if an *E. coli* strain likely had a type I or type II/III *cdtB* gene. We first tested the primers with several strains for which the sequence in the relevant region had already been determined. In every case, the type I primers, in combination with VAT2 or WMI1 (Table 2), amplified only sequences of the expected sizes from type I strains. Figure 2 shows the results for two known type I strains, 3061-99 and 3015-99. A PCR product was produced with only one of the two primer sets for each strain. For example, type I strain 3061-99 yielded a product with the type I primers (lane 2), but not with the type II/III primer set (lane 8). Similarly, the type II/III primers only amplified sequences of the expected sizes from known type II or type III strains (Table 1; Fig. 2, lane 11; and data not shown). We then tested the primer sets on eight additional CDT producers whose *cdtB* gene had not been sequenced. Of these strains, two produced a product with the type I primers (Table 2; Fig. 2, lanes 3 and 7) and the remaining six strains produced a PCR product only when tested with the type II/III primers (Table 2 and Fig. 2, lane 10, for strain 3084-98). All of the strains acted in a consistent and reproducible manner,

TABLE 2. CDT type-specific PCR of *E. coli* strains[a]

Strain	PCR				Sequence[b]
	Type I-F	Type II-F	Type I-R	Type II-R	
6468/62	+	−	+	−	Type I
3180-98	+	−	+	−	Type I
3015-99	+	−	+	−	Type I
3061-99	+	−	+	−	Type I
2P6	+	−	+	−	Type I
H19	+	−	+	−	Type I
3142-98	+	−	+	−	NS
3008-99	+	−	+	−	NS
9142-88	−	+	−	+	Type II
3032-96	−	+	−	+	Type III
3260-97	−	+	−	+	Type II
F6450-99	−	+	−	+	Type III
3148-97	−	+	−	+	NS
3084-98	−	+	−	+	NS
F5953-98	−	+	−	+	NS
3014-99	−	+	−	+	NS
F5971-99	−	+	−	+	NS
F6448-99	−	+	−	+	NS

[a] Four different sets of PCR primers were used: type I-F and WMI-1, type II-F and WMI-1, type I-R and VAT-2, and type II-R and VAT-2.

[b] Type I strains have partial CdtB sequences identical to that of strain 6468/62 (34). Type II strains have partial CdtB sequences identical or closely similar to that of 9142-88 (30), and type III strains have partial CdtB sequences identical or nearly identical to that of strain S5 (29). NS, not sequenced.

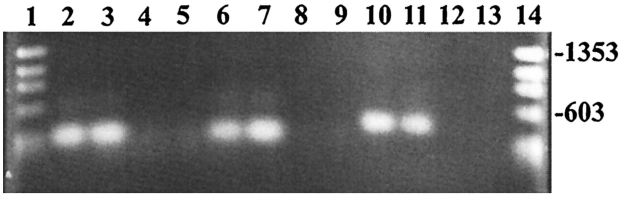

FIG. 2. PCR products showing CDT type specificity. Primers for lanes 2 to 7 were type I-1R and VAT-2. Primers for lanes 8 to 13 were type II-1F and WMI-1. Lanes: 1 and 14, *Hae*III ϕX174; 2 and 8, 3061-99; 3 and 9, 3142-98; 4 and 10, 3084-98; 5 and 11, 3260-97; 6 and 12, 3015-99; 7 and 13, 3008-99. Selected DNA fragment standard sizes, in base pairs, are shown to the right.

suggesting that these type-specific primers can be successfully used to differentiate type I from type II and type III CDT-producing strains. These primers cannot distinguish between type II and type III sequences.

Clark et al. (3) recently reported on the use of three sets of PCR primers for distinguishing between the three types of *cdt* genes. The PCR primers for detection and amplification of the type I and type II *cdt* genes were first reported by Okuda et al. (25) and are derived from the *cdtA* genes of the type I and type II prototype strains, 6468/62 and 9142-88, respectively. The type III primers are taken from the *cdtA* and *cdtC* genes of S5, such that the entire *cdt* coding region is amplified (3, 29). We tested these primers on our CDT-producing strains in order to determine if they would successfully detect and distinguish the different *cdt* sequences in these strains. The best results were achieved with the type I primers, although these were not without deficiencies. The type I strains 3008-99, 3015-99, 3061-99, H1, H19, and 2P6 all produced a single appropriately sized product (575 bp) with the type I primers. The results for type I strains 2P6, 3008-99, 3015-99, and 3061-99 are shown in Fig. 3A. However, type I strain 3180-98 produced no PCR product, and type I strain 3142-98 produced only a trace amount of product with these primers (Fig. 3A, lanes 9 and 8, respectively). No type II or type III *cdt* sequences were amplified by these primers.

The type II and type III reactions had additional problems. As expected, the type II primers successfully amplified the appropriate region of *cdtA* from 9142-88 (Fig. 3B, lane 11). In addition, these type II primers also produced a significant amount of amplified product from strains 3084-9 and 3102-97 (Fig. 3B, lanes 6 and 10). The type III primers amplified the *cdt* genes from strains F5953-98, F6450-99, and 3032-96 (Fig. 3C, lanes 5, 8, and 9) as well as from the prototype strain, S5 (lane 1). These results for both the type II and type III primers are in agreement with our sequencing results for strains 3102-97, 3032-96, and F6450-99; no *cdt* genes from strains F5953-98 and 3084-98 have been sequenced. However, neither the type II nor type III primers could amplify any product from the type II/III strain 3014-99 (Fig. 3B, lane 2, and data not shown). Similarly, the type II primers produced only faint products and the type III primers produced no products with the type II/III strains 3148-97, F5971-99, and F6448-99 (Fig. 3B and C). The faint products that these latter three strains produced, as well as the product produced from the known type II strain 3260-97, with the type II primers were less distinct than the product produced from strain S5, the prototype type III strain (Fig. 3B, compare lane 1 to lanes 3, 4, 7, and 9). Strain 3139-97, a known type III strain, produced no product with the type III primers (Fig. 3C, lane 7). In other words, 6 of the 12 type II or type III strains either were undetected by these primers or, in the case of some results with the type II primers, the amplified products appeared to be the result of non-type-specific amplification, since the amount of product was less than that produced by the type III control strain. These results indicated that the type II-specific primers are not specific and will amplify *cdt* sequences in some type III strains and that some type II and type III strains are not detected by either the type II or type III primers.

Correlation of strain characteristics and CDT type. Analysis of the strains' known virulence factors and serotypes in relation to CDT type revealed some interesting associations (Table 1). Of the eight strains identified as encoding type I CDT, five were serogroup O2 and, of these, three were O2 nonmotile strains. Three of the strains, H1, H19, and 2P6, were initially identified as CDT producers in a study by Johnson and Stell (17) of *E. coli* strains associated with urosepsis, and certain O2 serotypes are well documented to be relatively common causes of this type of extraintestinal infection. However, the other two O2 strains with a type I CDT were also not stool isolates; 3008-99 was isolated from the urine of a 77-year-old female, and strain 3015-99 was isolated from the blood of a 40-year-old female. The other three type I strains, 3180-98, 3061-99, and 3142-98, were all isolated from human stools but, in common with the O2 serogroup type I strains, they did not encode Shiga

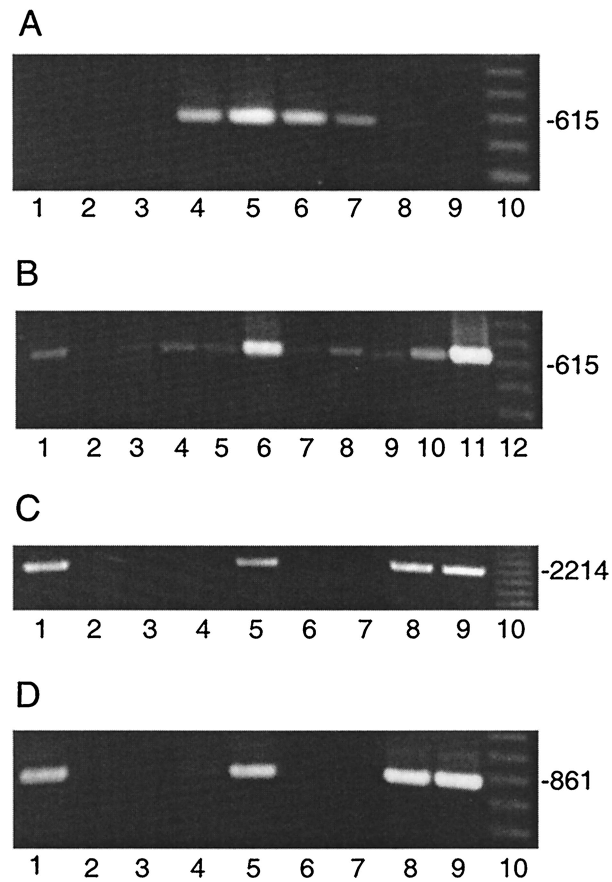

FIG. 3. PCR products produced by type I, type II, or type III CDT-producing strains. (A) PCR products amplified from the indicated *E. coli* strains with the type I-specific primers of Okuda et al. (25). Lanes: 1, 3014-99; 2, 3139-97; 3, F6450-99; 4, 2P6; 5, 3008-99; 6, 3015-99; 7, 3061-99; 8, 3142-98; 9, 3180-98; 10, 123-bp ladder. (B) PCR products amplified from *E. coli* strains with the type II-specific primers of Okuda et al. (25). Lanes: 1, S5; 2, 3014-99; 3, F5971-99; 4, F6448-99; 5, F5953-98; 6, 3084-98; 7, 3148-97; 8, 3032-96; 9, 3260-97; 10, 3102-97; 11, 9142-88; 12, 123-bp ladder. (C) PCR products amplified from *E. coli* strains with the type III-specific primers of Clark et al. (3). Lanes: 1, S5; 2, 9142-88; 3, F5971-99; 4, F6448-99; 5, F5953-98; 6, 3148-97; 7, 3139-97; 8, F6450-99; 9, 3032-96; 10, 123-bp ladder. (D) PCR products amplified from *E. coli* strains with CNF2-specific primers. Lanes: 1, S5; 2, 9142-88; 3, F5971-99; 4, F6448-99; 5, F5953-98; 6, 3148-97; 7, 3139-97; 8, F6450-99; 9, 3032-96; 10, 123-bp ladder. Sizes of relevant base pair markers are at the right.

toxin, enterohemolysin, or intimin. However, only the O2 serogroup type I strains possessed *pap* genes as assessed by PCR (reference 17 and data not shown). These results do not show that all CDT type I strains form a distinct clone, but they do indicate that at least a subset of type I CDT-producing strains appear to be associated with *E. coli* extraintestinal infections and that serogroup O2 strains that produce CDT likely possess type I CDT genes. In general, type I CDT-producing strains share the distinction of not having any of several known *E. coli* diarrheagenic-associated virulence markers (Table 1).

The type II CDT-producing strains varied from the type III strains based both on their *cdtB* fragment sequence and upon their complement of known virulence markers. There were three type III strains that encoded Stx1 and CNF2 but not intimin (Table 1; Fig. 3D); these are the same three strains that produced a product with the type III primers based on the *cdt* sequences in strain S5 (Fig. 3C). These strains appear to be similar to some of the bovine strains described by De Rycke et al. (7) in that they encode CDT type III and CNF2 but not F17 fimbriae (data not shown), although two of these three strains were isolated from humans. In any case, these strains appear to define a group of similar CDT-producing strains that make a type III CDT and also produce CNF2 and Stx1. The other known type III strain, 3139-97, encoded Stx1, Stx2, and the enterohemolysin. This strain was not identified by the type III-specific primers of Clark et al. (3) and thus appears to be distinct from the already-mentioned group of type III strains.

The three sequence-verified type II strains encode intimin, but apparently they encode no known *E. coli* toxins other than CDT (Table 1). In addition, one of the nonsequenced type II/III strains was identified as type II on the basis of a type II-specific PCR. This strain, 3084-98, also possesses an *eae* gene but none of the other virulence genes tested for in this work. None of these *eae*-carrying type II CDT producers tested positive for the presence of the EAF plasmid and, of note, all were nonmotile. These four strains appear to constitute another group of strains with significant commonalities, although their pathogenic significance is presently unknown. However, one of these CDT type II strains, 3260-97, is one of five strains that were described previously by Janda et al. (15) as either unusual biotypes of *E. coli* or as representatives of a new *Escherichia* species. All five of these strains were isolated from humans with diarrheal disease, and all five produce CDT (data not shown).

In addition to the six type II or III strains for which partial *cdtB* sequence data were obtained, six additional type II or III strains are shown in Table 1. Two of these strains, 3084-98 and F5953-98, were identified as to their CDT type based on the type II- and III-specific PCR methods discussed above (3). The other four strains have not yet been conclusively identified as to their CDT type, other than that they belong to either type II or type III (Tables 1 and 2). Three of these strains encode Stx2, and one of these also encodes Stx1. Our results from the sequenced strains would suggest that these strains are likely type III strains. The fourth strain identified as carrying either type II or III *cdt* genes, 3014-99, encoded neither intimin nor any toxins other than CDT in repeated PCR tests.

RAPD analysis. In an effort to learn more about the possible relatedness of strain 3014-99 to selected representatives of the different CDT types, RAPD analysis was performed. Each strain listed in Table 1 as well as some known siblings of selected strains were tested. These experiments are continuing, but our results, some of which are shown in Fig. 4, indicated that strain 3014-99, which has a type II/III partial *cdtB* sequence based on type-specific PCR, had a primer 2 RAPD pattern identical to that of several type I strains, including 3061-99, 3008-99, and 3015-99 (Fig. 4, lanes 5, 6, 7, and 8). The 3014-99 patterns achieved with primers 1 and 3 also appeared to be closely similar to that of the type I group, including strain 3008-99 (data not shown).

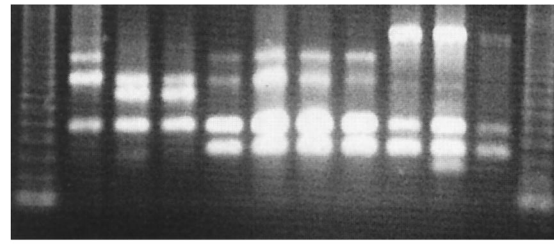

FIG. 4. RAPD analysis of CDT-producing *E. coli* strains, with an agarose gel (1%) of DNA fragments produced by RAPD using primer 2 and template DNA from the indicated strains. Lanes 1 and 12, 123-bp ladder (Invitrogen Corp.); lane 2, 3142-98 (type I); lane 3, 3180-98 (type I); lane 4, 3179-98 (a sibling of 3180-98); lane 5, 3061-99 (type I); lane 6, 3015-99; lane 7, 3008-99 (type I); lane 8, 3014-99 (type II/III); lane 9, F5971-99 (type II/III); lane 10, F5953-98 (type III); lane 11, 3139-97 (type III). The lowest rung of the 123-bp ladder is indicated at the right.

DISCUSSION

These studies were initiated in order to discover more about the relatedness of CDT-producing *E. coli* strains. The strains examined here were recovered from both gastrointestinal and extraintestinal infections. Partial sequence information derived from these strains indicated that *cdt* sequences can be broken down into two general categories: type I and type II/III. The type I strains studied here have CdtB partial sequences that are identical or nearly identical to that of strain E6468/62 reported by Scott and Kaper (34). The type II/III strains have CdtB partial sequences that are similar or identical to that of the type II and type III prototype strains 9142-88 and S5 (29, 30). We have grouped the type II and type III strains together because the *cdt* sequences of the prototype strains are closely related to each other (93 to 94% identical and conserved amino acids for all three Cdt subunits).

Overall, our isolates included several strains with CdtB partial sequences that were identical to the prototype type I and type III sequences, but two strains possessed CdtB sequences closely related, but not identical, to the type II and III sequences of both S5 and 9142-88. We have chosen not to assign these strains separate types (e.g., types IV and V) but rather to call them variants of the type II/III group, since their *cdt* genes have not been sequenced in their entirety and because it is not known if the Cdt proteins produced by these strains differ significantly in their antigenic specificities or in any other way.

It is possible that there are other substantially different types of *E. coli cdt* sequences, but the use of the degenerative primers VAT2 and WMI1 for our initial screen for the presence of *cdt* sequences was chosen in order to detect as many variants as possible. These primers are derived from regions of CdtB that are very highly conserved in all CdtBs and have been used to detect *cdt* sequences in several different genera (e.g., references 6 and 26).

While sequence data suggested two general groups of CDT producers, analysis of the strains' complements of virulence markers suggested that there were really at least three basic types of CDT producers. The type I CDT-producing strains analyzed here produced no other toxins besides CDT, with the exception of one strain that encoded cytotoxic necrotizing factor type 1 (CNF1). Several of the type I CDT strains were serogroup O2 and all of these possessed *pap* genes, indicating that these strains probably belong to the well-studied O2 group of extraintestinal (uropathogenic) strains. The other type I strains did not possess *pap* genes, and more study will be needed to determine whether these strains have a similar pathogenic potential.

Type II/III strains seemed to fall into two classes: those that make intimin but not any known toxins other than CDT, and those that make one or more Shiga toxins. Interestingly, the latter group included the three strains 3032-96, F6450-99, and 3139-97 whose partial CdtB sequences were identical to that of S5. PCR tests for other virulence markers indicated that the unsequenced strains F5953-98, 3148-97, F5971-99, and F6448-99 also belong to this group of strains that encode Shiga toxin. Three of these seven Shiga toxin-producing strains also encoded CNF2, suggesting a relationship with the CNF2 producers profiled by De Rycke et al. (7). However, only one of our CNF2-producing strains, F5953-98, was a bovine isolate, suggesting that at least some type III CDT-producing, CNF2-producing *E. coli* strains can affect humans.

On the other hand, the strains that encode intimin and CDT included 9142-88, the prototype type II strain, and the two strains, 3102-97 and 3260-97, that possessed the new variant CdtB sequences shown here (Fig. 1). In addition, strain 3084-98 can be included in this group since it encodes intimin and CDT, and it was identified as a type II strain by type-specific PCR (Fig. 3B). These strains, which we are calling type II strains, appear to form a group of CDT producers that possess similar virulence markers and that have related, but not necessarily identical, *cdt* sequences. The pathogenic potential of this group is unclear, but their identification as a subset of CDT producers should facilitate future studies aimed at determining CDT's role in *E. coli* disease.

On the genetic level, the variation seen in the type II strains' *cdt* sequences may reflect accumulated changes since acquisition of ancestral *cdt* genes, or it may mean that multiple transfers of closely related but nonidentical *cdt* sequences have occurred within this group of Eae-producing strains. The former seems a more likely hypothesis, given the similar complement of virulence-associated genes present in these strains. On the other hand, the *cdt* sequence identity found in the type III strains may argue for more recent acquisition of the *cdt* genes by these Shiga toxin-producing strains.

Strain 3014-99 presents an interesting exception to the general correlations described for the type I, II, and III strains. Based on PCR tests, this strain produces type II or III CDT, yet the overall genetic make-up of the strain, reflected in RAPD analysis, and also its lack of genes encoding either Shiga toxin or intimin appear to indicate that it is more closely related to type I CDT-producing *E. coli* than to the type II or III CDT producers. It will be of interest to further characterize both this strain as well as additional strains in related groups so that we can better understand the genetics, biology, and pathogenic potential of *E. coli* CDT-producing strains.

While preparing this report, a paper appeared by Clark et al. (3) which looked at the association of *cdt* genes with other virulence markers from selected groups of *E. coli* strains isolated from both animals and humans. Their results were somewhat similar to our correlations, but not identical. For example, they found that many type I-producing *E. coli* strains possessed no other known virulence markers, except that several type I strains also produced CNF1. They found that type III CDT producers were most often combined with either CNF2 or CNF2 and Stx1. These strains were generally derived from cattle, and thus the association with CNF2 and Stx1 is not surprising. Our greatest difference was in characterization of the type II and III strains. Our type II strains all contained *eae* genes, while only one of five of theirs did and it was in combination with *cnf1*. Also, two of our type III strains contained both stx_1 and stx_2 genes, while none of theirs did. In fact, only 2 of their 50 CDT producers contained an stx_2 gene, while four out of seven of our type III strains had an stx_2 gene. It should also be noted that their method of distinguishing the type of CDT differed from our methods. They used PCR primers based primarily on differences within the *cdtA* gene of the three prototype strains (18). In agreement with our studies of the *cdtB* gene from type III strains reported here, their type III sequences were identical to that of strain S5. (The *cdt* PCR products from type I and type II strains produced by these investigators based on sequences from strains 6468/62 and 9142-88, respectively, were not sequenced.) However, our data clearly indicate that sequences within the type II group can vary, and our use of their primers with our strains showed that, in fact, these primers did not detect or distinguish all CDT producers within the three CDT type groups. This in turn suggests that additional, as-yet-unsequenced variations exist and that use of degenerative primers for preliminary screening for CDT producers is warranted.

Our RAPD and sequence data support the notion that strains that produce a particular type of CDT may be more closely related to each other than to other *E. coli* CDT producers but that not all strains producing a particular type of CDT can be considered close relatives (e.g., type 1 strains 3008-99 and 3180-98). That is, strains that produce a particular type of CDT may comprise two or more clonal groups of *E. coli* pathogens. Our RAPD data also suggest that some of the *E. coli* CDT-producing strains, such as the type I strains 3008-99 and 3061-99, may be closely related to each other despite having different serotypes.

The results presented here are an attempt to begin defining what types of *E. coli* pathogens produce CDT. Our data indicate that a fairly defined subset of uropathogenic *E. coli* strains contain *cdt* genes, that additional extraintestinal isolates of uncertain clonal identity also can carry *cdt* genes, and that strains with at least some characteristics in common with Shiga toxin-producing *E. coli* and enteropathogenic strains can carry *cdt* genes. A recent report by Janka et al. (16) indicated that O157:H$^-$ strains and, rarely, O157:H7 strains may encode a type III-like CDT. In addition, Mainil et al. (24) recently described a variant of type I CDT which they termed type IV CDT and which was found in some CNF1-producing *E. coli* strains from a variety of sources. Additional combinations of *E. coli* virulence genes among CDT producers appear likely to exist given the variation seen here and that reported by others.

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REFERENCES

1. **Adu-Bobie, J., G. Frankel, C. Bain, A. G. Goncalves, L. R. Trabulsi, G. Douce, S. Knutton, and G. Dougan.** 1998. Detection of intimins α, β, γ, and δ, four intimin derivatives expressed by attaching and effacing microbial pathogens. J. Clin. Microbiol. **36:**662–668.
2. **Bertin, Y., C. Martin, E. Oswald, and J.-P. Girardeau.** 1996. Rapid and specific detection of F17-related pilin and adhesin genes in diarrheic and septicemic *Escherichia coli* strains by multiplex PCR. J. Clin. Microbiol. **34:**2921–2928.
3. **Clark, C. G., S. T. Johnson, R. H Easy, J. L Campbell, and F. G. Rodgers.** 2002. PCR for detection of *cdt*-III and the relative frequencies of cytolethal distending toxin variant-producing *Escherichia coli* isolates from humans and cattle. J. Clin. Microbiol. **40:**2671–2674.
4. **Comayras, C., C. Tasca, S. Y. Pérès, B. Ducommun, E. Oswald, and J. De Rycke.** 1997. *Escherichia coli* cytolethal distending toxin blocks the HeLa cell cycle at the G_2-M transition by preventing cdc2 protein kinase dephosphorylation and activation. Infect. Immun. **65:**5088–5095.
5. **Cope, L. D., S. Lumbley, J. L. Latimer, J. Klesney-Tait, M. K. Stevens, L. S. Johnson, M. Purven, R. S. Munson, Jr., T. Lagergård, J. D. Radolf, and E. J. Hansen.** 1997. A diffusible cytotoxin of *Haemophilus ducreyi.* Proc. Natl. Acad. Sci. USA **94:**4056–4061.
6. **Cortes-Bratti, X., C. Karlsson, T. Lagergård, M. Thelestam, and T. Frisan.** 2001. The *Haemophilus ducreyi* cytolethal distending toxin induces cell cycle arrest and apoptosis via the DNA damage checkpoint pathway. J. Biol. Chem. **276:**5296–5302.
7. **De Rycke, J., A. Milon, and E. Oswald.** 1999. Necrotoxic *Escherichia coli* (NTEC): two emerging categories of human and animal pathogens. Vet. Res. **30:**221–233.
8. **Elwell, C. A., and L. A. Dreyfus.** 2000. DNase I homologous residues in CdtB are critical for cytolethal distending toxin-mediated cell cycle arrest. Mol. Microbiol. **37:**952–963.
9. **Eyigor, A., K. A. Dawson, B. E. Langlois, and C. L. Pickett.** 1999. Cytolethal distending toxin genes in *Campylobacter jejuni* and *Campylobacter coli* isolates: detection and analysis by PCR. J. Clin. Microbiol. **37:**1646–1650.
10. **Franke, J., S. Franke, A. Schmidt, A. Schwarzkopf, L. H. Wieler, G. Baljer, L. Beutin, and H. Karch.** 1994. Nucleotide sequence analysis of enteropathogenic *Escherichia coli* (EPEC) adherence factor probe and development of PCR for rapid detection of EPEC harboring virulence plasmids. J. Clin. Microbiol. **32:**2460–2463.
11. **Fratamico, P. M., S. K. Sackitey, M. Weidmann, and M. Yi Deng.** 1995. Detection of *Escherichia coli* O157:H7 by multiplex PCR. J. Clin. Microbiol. **33:**2188–2191.
12. **Frisk, A., M. Lebens, C. Johansson, H. Ahmed, L. Svensson, K. Ahlman, and T. Lagergård.** 2001. The role of different protein components from the *Haemophilus ducreyi* cytolethal distending toxin in the generation of cell toxicity. Microb. Pathog. **30:**313–324.
13. **Hassane, D. C., R. B. Lee, M. D. Mendenhall, and C. L. Pickett.** 2001. Cytolethal distending toxin demonstrates genotoxic activity in a yeast model. Infect. Immun. **69:**5752–5759.
14. **Hassane, D. C., R. B. Lee, and C. L. Pickett.** 2003. *Campylobacter jejuni* cytolethal distending toxin promotes DNA repair responses in normal human cells. Infect. Immun. **71:**541–545.
15. **Janda, J. M., S. L. Abbott, and M. J. Albert.** 1999. Prototypal diarrheagenic strains of *Hafnia alvei* are actually members of the genus *Escherichia.* J. Clin. Microbiol. **37:**2399–2401.
16. **Janka, A., M. Bielaszewska, U. Dobrindt, L. Greune, M. A. Schmidt, and H. Karch.** 2003. Cytolethal distending toxin gene cluster in enterohemorrhagic *Escherichia coli* O157:H$^-$ and O157:H7: characterization and evolutionary considerations. Infect. Immun. **71:**3634–3638.
17. **Johnson, J. R., and A. L. Stell.** 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis. **181:**261–272.
18. **Johnson, W. M., and H. Lior.** 1988. A new heat-labile cytolethal distending toxin (CLDT) produced by *Escherichia coli* isolates from clinical material. Microb. Pathog. **4:**103–113.
19. **Lara-Tejero, M., and J. E. Galán.** 2000. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. Science **290:**354–357.
20. **Lara-Tejero, M., and J. E. Galán.** 2001. CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. Infect. Immun. **69:**4358–4365.
21. **Lee, R. B., D. C. Hassane, D. L. Cottle, and C. L. Pickett.** 2003. Interactions of *Campylobacter jejuni* cytolethal distending toxin subunits CdtA and CdtC with HeLa cells. Infect. Immun. **71:**4883–4890.
22. **Louie, M., J. De Azavedo, R. Clarke, A. Borczyk, H. Lior, and M. Richter.** 1994. Sequence heterogeneity of the *eae* gene and detection of verotoxin-producing *Escherichia coli* using serotype-specific primers. Epidemiol. Infect. **112:**449–461.
23. **Madico, G., N. S. Akopyants, and D. E. Berg.** 1995. Arbitrarily primed PCR DNA fingerprinting of *Escherichia coli* O157:H7 strains by using templates from boiled cultures. J. Clin. Microbiol. **33:**1534–1536.
24. **Mainil, J. G., E. Jacquemin, and E. Oswald.** 2003. Prevalence and identity of *cdt*-related sequences in necrotoxigenic *Escherichia coli.* Vet. Microbiol. **94:** 159–165.
25. **Okuda, J., H. Kurazono, and Y. Takeda.** 1995. Distribution of the cytolethal distending toxin A gene (*cdtA*) among species of *Shigella* and *Vibrio*, and cloning and sequencing of the *cdt* gene from *Shigella dysenteriae.* Microb. Pathog. **18:**167–172.
26. **Olsvik, O., E. Rimstad, E. Hornes, N. Strockbine, Y. Wasteson, A. Lund, and K. Wachsmuth.** 1991. A nested PCR followed by magnetic separation of amplified fragments for detection of *Escherichia coli* Shiga-like toxin genes. Mol. Cell. Probes **5:**429–435.
27. **Osek, J., P. Gallien, M. Truszczyñski, and D. Protz.** 1999. The use of polymerase chain reaction for determination of virulence factors of *Escherichia coli* strains isolated from pigs in Poland. Comp. Immunol. Microbiol. Infect. Dis. **22:**163–174.
28. **Pass, M. A., R. Odedra, and R. M. Batt.** 2000. Multiplex PCRs for identification of *Escherichia coli* virulence genes. J. Clin. Microbiol. **38:**2001–2004.
29. **Pérès, S. Y., O. Marchès, F. Daigle, J.-P. Nougayrède, F. Hérault, C. Tasca, J. De Rycke, and E. Oswald.** 1997. A new cytolethal distending toxin (CDT) from *Escherichia coli* producing CNF2 blocks HeLa cell division in G_2/M phase. Mol. Microbiol. **24:**1095–1107.
30. **Pickett, C. L., D. L. Cottle, E. C. Pesci, and G. Bikah.** 1994. Cloning, sequencing, and expression of the *Escherichia coli* cytolethal distending toxin genes. Infect. Immun. **62:**1046–1051.
31. **Pickett, C. L., E. C. Pesci, D. L. Cottle, G. Russell, A. N. Erdem, and H. Zeytin.** 1996. Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* genes. Infect. Immun. **64:**2070–2078.
32. **Pickett, C. L., and C. A. Whitehouse.** 1999. The cytolethal distending toxin family. Trends Microbiol. **7:**292–297.
33. **Reid, S. D., D. J. Betting, and T. S. Whittam.** 1999. Molecular detection and identification of intimin alleles in pathogenic *Escherichia coli* by multiplex PCR. J. Clin. Microbiol. **37:**2719–2722.
34. **Scott, D. A., and J. B. Kaper.** 1994. Cloning and sequencing of the genes encoding *Escherichia coli* cytolethal distending toxin. Infect. Immun. **62:**244–251.
35. **Sugai, M., T. Kawamoto, S. Y. Pérès, Y. Ueno, H. Komatsuzawa, T. Fujiwara, H. Kurihara, H. Suginaka, and E. Oswald.** 1998. The cell cycle-specific growth-inhibitory factor produced by *Actinobacillus actinomycetemcomitans* is a cytolethal distending toxin. Infect. Immun. **66:**5008–5019.
36. **Whitehouse, C. A., P. B. Balbo, E. C. Pesci, D. L. Cottle, P. M. Mirabito, and C. L. Pickett.** 1998. *Campylobacter jejuni* cytolethal distending toxin causes a G_2-phase cell cycle block. Infect. Immun. **66:**1934–1940.
37. **Young, V. B., K. A. Knox, and D. B. Schauer.** 2000. Cytolethal distending toxin sequence and activity in the enterohepatic pathogen *Helicobacter hepaticus.* Infect. Immun. **68:**184–191.
38. **Youssef, M., A. Shurman, M. Bougnoux, M. Rawashdeh, S. Bretagne, and N. Strockbine.** 2000. Bacterial, viral and parasitic enteric pathogens associated with acute diarrhea in hospitalized children from northern Jordan. FEMS Immunol. Med. Microbiol. **28:**257–263.

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